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Non-oncogenic T-region mutants of *Agrobacterium tumefaciens* do transfer T-DNA into plant cells

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Keywords: *Agrobacterium tumefaciens*, site-directed mutagenesis, non-oncogenic T-region mutants, T-DNA transfer

Abstract

A new procedure for site-directed mutagenesis has been applied to the shooting and rooting loci of T-DNA of an octopine Ti-plasmid of *Agrobacterium tumefaciens*. Mutants have been obtained which induced tumours that either developed shoots or produced more roots than normally observed. Double mutations, in which both types of T-DNA loci were affected, resulted in non-oncogenic strains. Indications have been obtained, showing that T-DNA coded oncogenic functions can be eliminated without affecting T-DNA transfer into plant cells.

Introduction

Agrobacterium tumefaciens, the causative agent of the plant tumour crown gall, harbours a large plasmid (the Ti-plasmid) which is essential for its capacity to induce tumours (25, 26, 29). Homology to a segment of the Ti-plasmid is detected with DNA from tumour cells, but not with DNA from normal cells (2, 3, 24). This part of the Ti-plasmid, which is called T-DNA, is integrated into nuclear DNA of plant tumour cells, where it is transcribed into polyadenylated RNAs, of which at least some are capped and translated into protein (23, 27, 28). Octopine type *A. tumefaciens* strains induce tumour cells that synthesize octopine which can subsequently be utilized by the bacterium because the Ti-plasmid carries genes for the catabolism of this compound. The phytopathogenic interaction between *A. tumefaciens* and plant cells has therefore been named 'genetic colonization' (22), and represents successful natural genetic engineering of a eukaryotic organism by a prokaryote.

Some tumours induced by octopine type *A. tumefaciens* strains appear to contain two Ti-plasmid segments. In addition to T_L-DNA, which is always found in octopine tumour tissues, a second

fragment called T_R-DNA can be present, linked to T_L-DNA or independently integrated into the plant genome (24). The T_R-DNA neighbours the T_L-DNA on the physical map of the Ti-plasmid and can exist in a high copy number per tumour cell. Up to 20 copies of T_R-DNA have been observed, whereas T_L-DNA occurs in one to a few copies. Agrobacteria harbouring a Ti-plasmid lacking the T_R-region, but with an intact T_L-region can normally induce tumours, indicating that no functions essential to tumour induction are located on the T_R-region (17). If, however, the T_L-region on the Ti-plasmid is deleted, agrobacteria can no longer induce tumours (12), suggesting that the oncogenes are located on T_L-DNA.

Apart from the T-region, another segment on the Ti-plasmid is required for virulence of the bacterium (5, 15). Various mutations in this region are complementable *in trans* by R-prime plasmids, carrying the corresponding wild-type region of the Ti-plasmid (7, 9, 11). It is likely, therefore, that this area, which is called Vir-region, has to be expressed in the bacterium for virulence. As yet, no specific functions are ascribed to the Vir-region.

Mutagenesis of the T_L-region of an octopine Ti-plasmid has identified a number of different loci

(16). Mutations at the left part or in the middle of the T_L-region result in bacteria that induce tumours with an increased capacity to stimulate the development of shoots or roots, respectively. Mutations in the T_L-region have also been described which eliminate the ability of tumour cells to synthesize octopine. More recently, site-directed mutagenesis has been applied to the T_L-region in such a way that with a maximum interval of 400 basepairs an insertion mutation is introduced revealing four separated loci (6). No single insertion mutation is found which caused complete loss of oncogenicity.

The mechanism by which T-DNA is transferred and integrated into the plant genome is unknown. In general, T-DNA is a discrete entity suggesting that border sequences of the T-region are important in the tumour induction process. In this paper we report results that are obtained with a new efficient procedure for site-directed mutagenesis of an octopine Ti-plasmid in *E. coli* (10). Mutations were directed towards the different T-DNA loci and its border sequences. Moreover, double mutations affecting different T-DNA loci have been studied. Transfer of T-DNA from non-oncogenic strains, obtained in these studies, are discussed.

Materials and methods

Bacteria

Bacterial strains are listed in Table 1. Media, conjugation conditions and selection of transconjugants have been described elsewhere (9).

Plasmids

Recombinant plasmids, used in this work, are shown in Table 2. Constructions for site-directed mutagenesis of Ti-plasmids are outlined in Table 3. Plasmids were isolated from *E. coli* as described by Birnboim & Doly (1), and from *A. tumefaciens* as described by Koekman *et al.* (13).

Restriction endonuclease digestion, agarose gel electrophoresis and Southern blot hybridization

These were all carried out as described by Prakash *et al.* (19).

Cloning procedures

These were performed as previously reported (10).

Assay for tumour induction

A sterile wooden toothpick was dipped in a colony of the strain to be tested and then used to puncture the stem of tomato or sunflower. On Kalanchoë stems, samples of overnight cultures were inoculated 24 h after wounding. For *Nicotiana rustica* the stem was first wounded and then the bacteria were introduced. Aseptically grown shoots of *N. tabacum* were tested by dipping a sterile needle in a colony and puncturing the stem of the shoots.

Table 1. Bacterial strains.

	Chromosomal markers	Plasmids	Relevant plasmid markers	Source
<i>A. tumefaciens</i> strains				
(LBA No.)				
288 ^a	Rif, Nal	—		J. Schell
2402	Rif, Nal, Rec	—		Molbas, unpublished
969	Rif, Nal	pAL969	Km, occ ⁺ , onc ⁺	Hooykaas <i>et al.</i> (1980)
1820	Gen	pAL1820	Km, Spc, Cb, occ ⁺ , onc ⁺	Hille <i>et al.</i> (1982)
<i>E. coli</i> strains				
KMBL 1164	Pro, Thi	—		P. v.d. Putte
KMBL 1001	—	—		P. v.d. Putte

^a All *A. tumefaciens* strains used in this work have an LBA288 genetic background, unless stated otherwise.

Table 2. Recombinant plasmids.

Vectors	Relevant plasmid encoded markers	Source
pTR262	insertional activation of Tc	T. Roberts
pACYC184	Tc, Cm	A. Chang
pRL263	Em, Cm	J. Hille, unpublished

T-region clones	Vector	T-region fragment
pRAL3503	pTR262	HindIII-1 (pTiAch5)
pRAL3501	pACYC184	EcoRI-7 (pTiAch5)
pRAL3502	pRL263	EcoRI-7 (pTiAch5)
pRAL3504	pACYC184	BamHI-8 (pTiAch5)

Results

Ti-plasmids of *A. tumefaciens* do not replicate in *E. coli*. Recently we described a cointegrate plasmid between the wide host range *IncP-1* type plasmid R772 and an octopine Ti-plasmid (pTiB6). This cointegrate plasmid, named pAL969, replicates in both *E. coli* and *A. tumefaciens*, and is perfectly

stable in both species. *Agrobacteria*, harbouring this plasmid have a normal capacity to induce tumours on several plant species. With the use of this cointegrate plasmid an easy and rapid procedure for site-directed mutagenesis of the Ti-plasmid in *E. coli* has been developed (10). In summary, this procedure has the following steps. In *E. coli* a mutagenized Ti-plasmid fragment cloned on an *E. coli* vector is brought together with the cointegrate plasmid pAL969. The resulting strain is subsequently used as a donor in a cross with an *E. coli* strain devoid of any plasmids. Transconjugants were selected on the base of transfer of the mutation. This can only have occurred by a cointegrate-mediated mobilization of the complete mutagenized Ti-plasmid clone, or by uptake of the mutation into the homologous region of the Ti-part of the cointegrate through double cross-over. In the latter case site-directed mutagenesis of the cointegrate plasmid has taken place.

Site-directed mutagenesis

Several site-directed mutations were made into

Table 3. T-region mutations.

Mutated T-region clone	Derived from T-region clone	Mutation located in T-region fragment	Mutation	pAL number of Ti-plasmid carrying the described mutation ^a
pRAL3511	pRAL3501	EcoRI-7	0.5 Mdalton PstI fragment substituted for a 2.7 Mdalton PstI fragment from pRL220 ^b carrying a Cm determinant.	pAL1831
pRAL3512	pRAL3503	HindIII-1	EcoRI fragments Ω and 19a substituted for a 5.8 Mdalton fragment from pRL 220 carrying a Cb and Cm determinant.	pAL1832
pRAL3513	pRAL3504	BamHI-8	Substitution of BglII fragments for an 0.9 Mdalton BamHI fragment containing a Cb determinant.	pAL1840
pRAL3514	pRAL3502	EcoRI-7	Substitution of BamHI fragments 28 and 30b for an 0.9 Mdalton BamHI fragment containing a Cb determinant.	pAL1844
pRAL3515, 3516, 3517	pRAL3502	EcoRI-7	Insertion of an 0.9 Mdalton BamHI fragment carrying a Cb determinant left of BamHI 30b, between 30b and 28, and right of 28, respectively.	pAL1845, 1847, 1848
pRAL3518	pRAL3502	EcoRI-7	Substitution of BamHI fragment 28, 150bp BamHI-PstI fragment and the 0.5 Mdalton PstI fragment for a BamHI-PstI fragment carrying a Cm determinant.	pAL1850

^a See also Fig. 2.

^b pRL220: see Fig. 1.

the T-region of the cointegrate plasmid in *E. coli* (see Fig. 2 and Table 3). The mutated cointegrates were transferred to *A. tumefaciens* in order to study the phenotype of the mutations with respect to plant tumour induction. The observed changes in tumour phenotype and oncogenicity are shown in Table 4. In the case of strain LBA1832 (pAL1832) the right border of T_L-DNA, which is located in *EcoRI* fragment 19a, was deleted. This did not result in a change of the tumour-inducing capacity, indicating that this border is not essential for tumour induction. In this mutant also the locus for octopine synthase was removed, which shows that this locus has no direct function in tumour induction or development. All tumours on tobacco analyzed, induced by this mutant, contain agropine, indicating the presence of T_R-DNA in these tumour cells. Upon infection of tobacco and *Kalanchoë* with strains LBA1831 (pAL1831) and LBA1848 (pAL1848) mainly roots developed. The mutation in pAL1848 is well localized in the *tmr* locus (tumour morphology roots). However, the number of loci that are mutated in pAL1831 is less well known, since part of the *tmr* locus is removed, though the *tml* locus is possibly also affected. At the transcriptional level the mutation in pAL1848 is located at the position of transcript 4 while the mutation in pAL1831 is at the position of transcript 4 and possibly also of transcript 6. In the case of LBA1850 (pAL1850) the entire *tmr* locus was deleted. The phenotype of the tumour induced by this mutant is

Table 4. Effect of T-region mutations on tumour induction.

Strain	Plasmid	Tumour induction tested on ^a	
		Tomato	Tobacco
LBA1832	pAL1832	++	++
LBA1831	pAL1831	+	+, roots
LBA1848	pAL1848	+	+, roots
LBA1847	pAL1847	++	++
LBA1850	pAL1850	+	+, roots
LBA1845	pAL1845	+/-	+, shoots
LBA1851	pAL1851	+/-	+, shoots
LBA1840	pAL1840	-	+, shoots

^a Different tumour morphologies are most pronounced on tobacco plants.

++, full size tumour induction;

+, tumour induction but only small tumours are formed;

+/-, weak tumour induction;

-, no tumour induction.

essentially the same as observed for an insertion mutation (LBA1848 (pAL1848)) and a small deletion (LBA1831 (pAL1831)) in the *tmr* locus (see Table 4).

A mutation in the *tms* locus at the position of transcript 1 (like in pAL1845), resulted in tumours on tobacco that developed shoots. The same was found when the positions of both transcripts 1 and 2 are mutated (like in pAL1851). A deletion overlapping the *tms* locus and eliminating transcripts 5, 7, 2 and 1 gave rise to strains which induced tumours with shoots, but after a long delay. Normally tumour development could be observed after two weeks, but in this case it took about eight weeks.

A site was found between the *tmr* and *tms* locus, in which insertions did not disturb the expression of known oncogenes (LBA1847 (pAL1847), see Fig. 2). This site, a *Bam* HI site, is located between the positions of transcripts 1 and 4. Bacteria harbouring a Ti-plasmid with a mutation 500 bp left of this site, induced a shooting tumour phenotype (like LBA1845), whereas those 1 000 bp to the right gave a rooting tumour phenotype (like LBA1848, see Fig. 3).

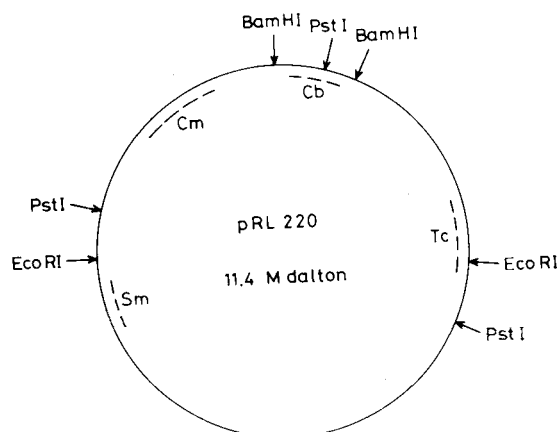


Fig. 1. Map of the plasmid pRL220. Restriction fragments of pRL220 have been used for mutagenesis of T-region clones (see Table 3):

Restriction fragment	Size (Mdalton)	Resistance determinant
BamHI	0.9	Cb
PstI	2.7	Cm
EcoRI	5.8	Cm, Cb

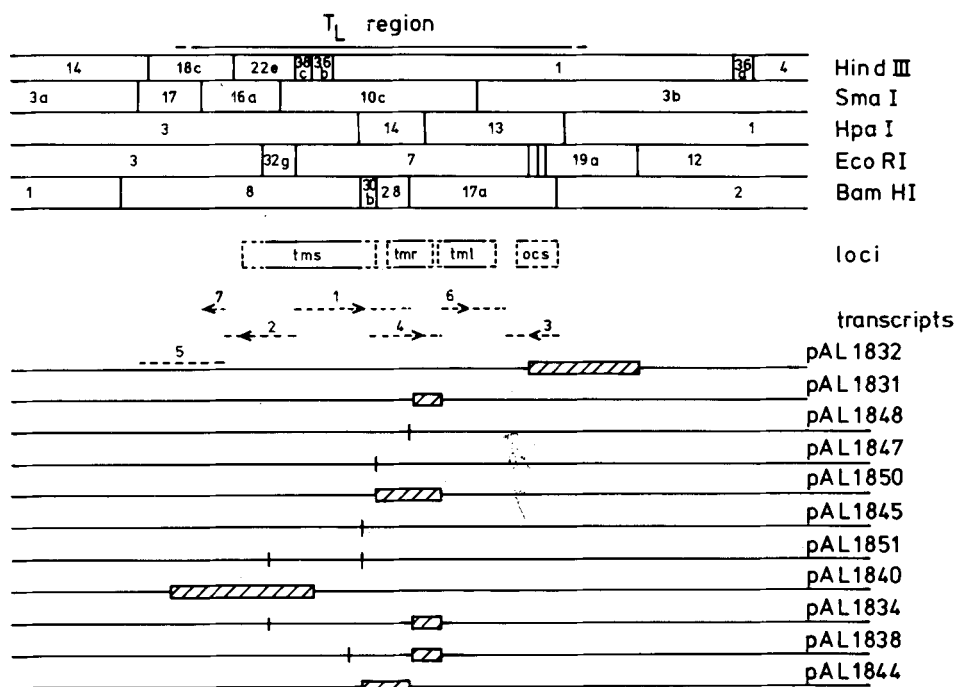


Fig. 2. T-region mutations. In the upper part a physical map of the T-region of an octopine Ti-plasmid (pTiB6) is shown for the restriction endonucleases *Hind*III, *Sma*I, *Hpa*I, *Eco*RI and *Bam*HI (4). The four different loci on T_L -DNA are indicated with boxes (6), and transcripts found in the plant tumour cell, including their polarity with arrows (28). Below, mutations introduced into the T-region are shown with their corresponding plasmid numbers. Deletions are indicated with shaded boxes and insertions by a vertical stripe. For cloning details see Table 3. Abbreviations: *tmr*, *tms* and *tml* stands for tumour morphology roots, shoots and large, respectively; *ocs* stands for octopine synthase.

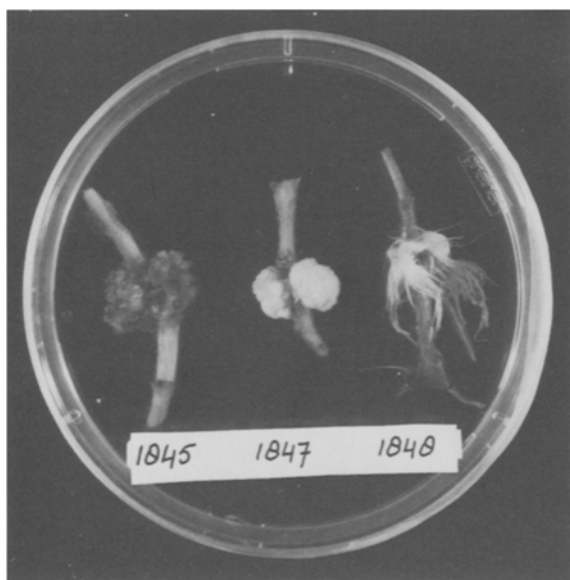


Fig. 3. Tumour induction by mutant *Agrobacterium* strains. Tumours were raised on tobacco and scored four weeks after infection. From left to right: LBA1845 (pAL1845), a shooter mutant interrupted at the position of transcript 1; LBA1847 (pAL1847), a normal oncogenic mutant, mutated between the positions of transcripts 1 and 4; LBA1848 (pAL1848), a rooter mutant interrupted at the position of transcript 4 (see also Fig. 2).

Mutations affecting both *tmr* and *tms*

On the assumption that the *tmr* and *tms* loci consist of genes that promote auxin-like and cytokinin-like activities, respectively, it may be expected that elimination of one of these activities will result in a rooting or shooting phenotype of the tumour. If both these activities are expressed normally an amorphous unorganized tumour will develop. However, if both activities are not expressed one can envisage that tumour development will not occur.

We had the availability of two mutants carrying one copy of the transposon Tn1831, coding for resistance against Sp, Sm and HgCl₂, inserted into the *tms* locus at the position of transcript 2 and transcript 1, respectively (Paul Hooykaas, pers. commun.). Through homologous recombination these single mutations were introduced separately into plasmid pAL1831, which was already mutated at the position of transcript 4 (and possibly 6), resulting in plasmids pAL1834 and pAL1838 (see Fig. 2). Plasmid pAL1834 now was mutated at the positions of transcripts 2, 4 and possibly 6, and plasmid pAL1838 at transcripts 1, 4 and possibly 6. A

plasmid was also constructed in which the region covering transcripts 1 and 4 was deleted (plasmid pAL1844, see Fig. 2). The position of the various mutations was verified by Southern blot hybridization, using the mutated plasmid DNA and T-region probes (Data not shown). Table 5 shows the results of plant tumour induction tests. Tumour induction assayed on several plant species was not observed with any of the described mutants (except for one, see discussion). This is in good agreement with a model proposing the presence of genes for auxin- and cytokinin-like activities on T-DNA.

T-DNA transfer

When manipulating plant cells by using the Ti-plasmid as a vector, it is of fundamental importance to know whether the activity of oncogenes on T-DNA can be suppressed without affecting the transfer, integration and expression of T-DNA into plant cells. Since we had the availability of non-oncogenic strains from this study, we were able to investigate this question. To this end we had to solve the problem of recognizing transformed plant cells, since they will not have a tumourous character. However, mixed infection of plants with mutant *A. tumefaciens* strains might result, through complementation, in a visual response. This has been demonstrated earlier by using a 1:1 mixture of a *tms* and a *tmr* mutant for tumour induction, which resulted in normal tumour development (18). Complementation could have occurred in the tumour within one cell, or between cells, each of which carried a different mutated T-DNA. The occurrence of the first possibility has been supported

by studying cloned tumour tissues (18). We assumed that a comparable complementation could also occur with appropriate non-oncogenic strains, if indeed the mutated T-DNAs are transferred and integrated into the plant genome. Appropriate *A. tumefaciens* strains are those which are mutated at the position of different transcripts within the same locus of T-DNA. Therefore, strain LBA2402 (pAL1834), mutated at the positions of transcripts 2, 4 and possibly 6, was mixed 1:1 with strain LBA2402 (pAL1838), mutated at the positions of transcripts 1, 4 and possibly 6. The mixture was inoculated on tobacco shoots. In this case one might expect the development of tumours with a morphology comparable to those induced by a strain carrying a mutation at the positions of transcripts 4 and possibly 6. Indeed, this was observed, but the size of the tumour and the number of roots was less than in the case of a mutant which had been mutated only at the positions of transcripts 4 and possibly 6, as in LBA1831 (pAL1831). To test whether transformation had occurred, the area surrounding the wound site was cut out and tested for octopine synthase activity. This was found to be positive and consequently demonstrated that T-DNA had been transferred and had most likely been integrated into the plant cells. Whether both T-DNAs had been transferred into one plant cell, in which complementation of oncogenes occurs within one cell, or different plant cells were transformed separately with the different T-DNAs, in which complementation occurs between plant cells, could not be established in this experiment. Nor could a combination of these possibilities be excluded.

A second indication that T-DNA is transferred by such non-oncogenic strains came from an experiment in which a normal oncogenic *A. tumefaciens* strain, carrying a deletion over the octopine synthase locus (LBA2402 (pAL1832)), was mixed 1:1 with a non-oncogenic strain, carrying an intact octopine synthase locus (LBA2402 (pAL1834)). The mixture was used to induce tumours on tomato. A plant tumour induced by *A. tumefaciens* often only partly consists of really transformed plant cells. The majority of cells can be normal and are stimulated to proliferate by neighbouring tumour cells (21). Therefore, we reasoned that after mixed infection plant cells that probably would have acquired T-DNA of the non-oncogenic strain (harbouring an intact *ocs*-locus) would proliferate in the develop-

Table 5. Double mutations in the T-region.

Strain	Plasmid	Positions of mutations (transcripts)	Tumour induction ^a
LBA1834	pAL1834	2, 4, (6)	–
LBA1838	pAL1838	1, 4, (6)	–
LBA1844	pAL1844	1, 4	– ^b

^a Tumour induction has been tested on the following plant species: *Nicotiana tabacum*, *Nicotiana rustica*, *Kalanchoë daigremontiana*, sunflower and tomato.

–, no tumour induction.

^b A very weak reaction could be observed only on *Nicotiana rustica*.

ing tumour induced by the oncogenic strain (having a deletion over the *ocs*-locus). Tumours raised on tomato appeared to be positive for octopine synthase activity, which demonstrated that the non-oncogenic bacteria had indeed delivered T-DNA into plant cells.

The third indication for T-DNA transfer from non-oncogenic strains came from a straightforward experiment. Strain LBA1834 (pAL1834) was inoculated on the basal end of a stem segment of a tobacco shoot. The resulting wound tissue that grew on the basal end of the segment when placed basal end up in culture medium was found to be positive for octopine synthase activity. This clearly demonstrates T-DNA transfer from this non-oncogenic strain. With strain LBA1838 (pAL1838), the same result was obtained.

Discussion

Using a simple and efficient new procedure several mutations have been introduced into the T-region of an octopine Ti-plasmid in *E. coli*. The mutated Ti-plasmids were conjugatively transferred to *A. tumefaciens* and tested for tumour induction on different plants.

Mutations affecting both tmr and tms

Mutations, altering both the *tmr* locus as well as the *tms* locus, gave rise to bacteria that were no longer able to induce tumours. This was found for mutations that affected the positions of transcripts 2, 4 and possibly 6 (as in pAL1834), and 1, 4 and possibly 6 (as in pAL1838). Tumour induction was tested on various plant species, but in no case could any reaction be observed. A strain harbouring a Ti-plasmid mutated at the positions of transcripts 1 and 4, like LBA1844 (pAL1844), did give a weak reaction on *N. rustica*, although no reaction could be observed on the other plant species tested. The small difference in tumour inducing capacity between LBA1838 (pAL1838) and LBA1844 (pAL1844), when tested on *N. rustica*, could indeed indicate that in pAL1838, in addition to the position of transcripts 1 and 4, probably also 6 had been affected. Mutations at essentially the same position as in LBA1844 (pAL1844) have been described (14, 20). In one case (14), a strain harbouring such a

mutated Ti-plasmid was reported to be tumorigenic on tobacco and tomato, although it took a long time to produce a small tumour. This result is somewhat contradictory to (20) and to our results, since with this *Agrobacterium* strain, LBA1844 (pAL1844), no reaction could be observed on these plant species even after several months. At present we do not have an explanation for this difference.

T-DNA transfer

We have tried to answer the question whether non-oncogenic bacteria, harbouring a Ti-plasmid with a mutated T-region, still transfer T-DNA into plant cells. This was done firstly by looking for complementation of oncogenes leading to an altered tumour phenotype, and secondly by looking for expression of a T-DNA encoded gene product. In both cases indications for T-DNA transfer to the plant cell were obtained. However, mixed infections were performed to obtain these results. The possibility of homologous recombination between the different types of mutated plasmids, when they happen to be together in one bacterium through plasmid exchange, can be excluded, because the bacteria carried a Rec mutation. But recombination within a plant cell that has acquired both mutated T-DNAs cannot be excluded. Therefore, the third approach is the most direct one. Non-oncogenic bacteria were inoculated on tobacco stem segments and the resulting wound tissue was subsequently analyzed for octopine synthase activity. In this case, recombination events within the bacterium or within the plant cells can be excluded. The presence of octopine synthase activity in tissue induced by strain LBA1834 or by strain LBA1838 directly demonstrates that these completely non-oncogenic strains have retained the capacity to transfer T-DNA to plant cells. This makes such strains valuable for transformation of plant cells with retention of the full morphogenic potential and a good basis for genetic manipulation of plants.

The right border of T_L-DNA

The right border of T_L-DNA was shown to be dispensible in tumour induction. Strains carrying a Ti-plasmid with a substitution for the right border of T_L-DNA and part of the locus for octopine synthase (LBA1832 (pAL1832)) had not lost their

capacity to induce normal tumours. This might be explained by assuming that this border has no role in tumour induction at all, or that another border is present and is now used. The second assumption is supported for two reasons. Firstly, indications are found that tumours on tobacco, induced by this strain, contain T_R -DNA. This was concluded from the presence of agropine, which is a function on T_R -DNA, in the tumours. T_R -DNA generally is not found in tumours raised on tobacco by octopine wild-type *A. tumefaciens* strains. Thus instead of using the right border of T_L -DNA (which has been deleted in the mutant), the right border of T_R -DNA might have been recognized now, which would mean that in such tumours T_L - and T_R -DNA are contiguous. Secondly, a deletion removing T_R -DNA and the right border of T_L -DNA renders the bacterium weakly oncogenic (17). Since it has been established that T_R -DNA does not contain genes that are essential in oncogenesis, the most likely explanation for its presence in tumours induced by our mutant is that it contributes a T_R -DNA border sequence.

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